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(54) Title: SYNERGISTIC COMPOSITION COMPRISING RAPAMYCIN AND CALCITRIOL

(57) Abstract

This invention provides a combination of rapamycin and 1,25–dihydroxy–cholecalciferol ($1,25(OH)_2D_3$) which is useful for inducing immunosuppression, and in the treatment of transplantation rejection, graft vs. host disease, autoimmune diseases, diseases of inflammation, adult T–cell leukemia/lymphoma, neoplasms, fungal infections, and hyperproliferative vascular disorders.

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SYNERGISTIC COMPOSITION COMPRISING RAPAMYCIN AND CALCITRIOL

BACKGROUND OF THE INVENTION

Recently, it has been recognized that numerous diseases or disorders can be prevented or treated through the use of immunosuppressive agents. In particular, through the use of immunosuppressive agents, organ transplantation has become a successful method of treating many serious disease states that otherwise might be fatal. What was once only an experimental procedure used in emergency life-threatening situations, is now used early in the care of patients with severe chronic diseases. Currently, the kidney, heart, lung, liver, bone marrow, pancreas (islet cells), cornea, small bowel, and skin are among allografts that are routinely performed. Additionally, xenografts are now performed using porcine heart valves.

Despite the advances made in the field of organ allografting, transplantation rejection remains the predominate factor leading to graft failure. Overall, graft rejection is a complex process involving the immune system. This process is briefly outlined below. Rejection appears to be initiated by blood borne antigen presenting cells (dendritic cells and monocytes expressing class II MHC molecules) of the allograft which migrate from the allograft. Antigen recognition and production of IL-1 by the antigen presenting cells causes the activation of $CD4^+$ T-cells thereby initiating an immune response leading to the ultimate graft rejection. Activated $CD4^+$ T-cells produce IL-2 which is a growth factor essential to the activation of both $CD8^+$ T-cells and B-cells. Clonal proliferation and maturation of alloantigen reactive cells leads to the production of effector T-cells (cytotoxic $CD8^+$ T-cells and $CD4^+$ T-cells) which migrate from the host lymphoid tissue and infiltrate the graft tissue. Infiltration involves initial adherence of the T-lymphocytes to vascular endothelium, transmigration through the vascular wall, migration within the graft, selective retention of activated cells within the graft, and local proliferation of cells. Antigen presenting graft cells are destroyed directly by cytotoxic $CD8^+$ T-cells. Additionally, $CD4^+$ T-cells produce other lymphokines such as interferon-γ (IFN-γ), IL-4, and IL-5 which also contribute to graft destruction. IFN-γ induces increased expression of HLA-A, -B, and -DR on graft tissue making it more vulnerable to effector mechanisms. IFN-γ also activates macrophages to initiate a delayed hypersensitivity reaction causing nonspecific damage to the graft. IL-4 and IL-5 are implicated in inducing antibody production by plasma cells leading to antibody mediated damage of the graft. [Hutchinson, I.,

Transplantation 3: 722 (1991); Garovoy, M.R., Basic and Clinical Immunology, ed. Stites, 7th ed., 747 (1991)].

Currently, allograft rejection is controlled using agents which suppress the immune response such as prednisone, methylprednisolone, azathioprine, cyclophosphamide, cyclosporin, antilymphocyte globulin, monoclonal antibodies, and irradiation. Of the chemotherapeutic agents currently used cyclosporin A is the most powerful and most frequently used, but has the unsatisfactory side-effect of nephrotoxicity in man, which can lead to structural renal damage.

Recently, rapamycin, a macrocyclic triene antibiotic produced by Streptomyces hygroscopicus [U.S. Patent 3,929,992] has been shown to prevent the formation of humoral (IgE-like) antibodies in response to an albumin allergic challenge [Martel, R., Can. J. Physiol. Pharm. 55: 48 (1977)], inhibit murine T-cell activation [Staruch, M., FASEB 3: 3411 (1989)], prolong survival time of organ grafts in histoincompatible rodents [Morris, R., Med. Sci. Res. 17: 877 (1989)], and inhibit transplantation rejection in mammals [U.S. Patent 5,100,999]. Rapamycin has also been shown to be useful in treating pulmonary inflammation [U.S. Patent 5,080,899], systemic lupus erythematosis [U.S. Patent 5,078,899], immunoinflammatory skin disorders, such as psoriasis [U.S. Patent 5,286,730], immunoinflammatory bowel disorders [U.S. Patent 5,286,731], ocular inflammation [U.S. Patent 5,387,589], hyperproliferative vascular disorders, such as restenosis [U.S. Patents 5,512,781 and 5,288,711], carcinomas [U.S. Patent 5,206,018 and 4,885,171], and cardiac inflammatory disease [U.S. Patent 5,496,832]; and in preventing the onset of insulin dependent diabetes mellitus [U.S. Patent 5,321,009]. Additionally, rapamycin has been shown to be useful in treating adult T-cell leukemia/lymphoma [European Patent Application 525,960 A1].

Besides its classical role in bone metabolism and in calcium / phosphate homeostasis, the active form of vitamin D, 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), also exhibits non-calcemic effects [Walters, M.R., Endocr. Rev. 13: 719 (1992)]. For example, $1,25(OH)_2D_3$ has a strong impact on the immune system, probably exerted via specific receptors present in both monocytes and activated T lymphocytes [Rigby, W.F.C., Immunol. Today 9: 54 (1988)].

$1,25(OH)_2D_3$ seems to impair immune cell interaction at the macrophage as well as at the T helper level [Rigby, W.F.C., Blood 76: 189 (1990)], and modulates the paracrine function of the two cell types [Manolagas, S.C., Semin. Nephrol. 14: 129 (1994)]. The direct inhibition by $1,25(OH)_2D_3$ of key interleukin secretion, such as

IL-2 [Muller, K., Immunol. Lett. 35: 177 (1993)], IFN-γ [Reichel, H., Proc. Natl. Acad. Sci. USA 84: 3385 (1987)] or IL-12 [Lemire, J.M., in Vitamin D, a pluripotent steroid hormone: structural studies, molecular endocrinology, and clinical applications, A. W. Norman ed, de Druyter, W. Berlin, p. 534 (194)], impedes further maturation and recruitment of other immune effectors.. These in *vitro* data are further supported by animal experiments. Indeed, $1,25(0H)_2D_3$ and some of its analogues, can prevent or diminish the severity of several autoimmune diseases, and prolong skin or heart allograft survival [Lemire, J.M., J. Cell Biochem. 49: 26 (1992)]. The treatment with vitamin D related drugs at therapeutically efficient doses is however accompanied by toxic hypercalcemic effects [Binderup, L., Biochem. Pharmacol. 43: 1885 (1992)].

DESCRIPTION OF THE INVENTION

This invention provides a combination of rapamycin and 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) which is useful for inducing immunosuppression, and in the treatment of transplantation rejection, graft vs. host disease, autoimmune diseases, diseases of inflammation, adult T-cell leukemia/lymphoma, neoplasms, fungal infections, and hyperproliferative vascular disorders.

When administered for the treatment or inhibition of the above disease states, the combination of rapamycin and $1,25(OH)_2D_3$ can be administered to a mammal orally, parenterally, intranasally, intrabronchially, transdermally, topically, intravaginally, or rectally.

This invention also provides a pharmaceutical composition comprising rapamycin and $1,25(OH)_2D_3$, and a pharmaceutical carrier.

The ability the combination of rapamycin and $1,25(OH)_2D_3$ to act as an immunosuppressive agent was established in an in vitro and in vivo standard pharmacological test procedure. The in vitro procedure evaluated the ability of the rapamycin and $1,25(OH)_2D_3$ combination to inhibit PHA-stimulated PMBC proliferation. The in vivo test procedure measured the ability of the rapamycin and $1,25(OH)_2D_3$ combination to inhibit the development of experimental allergic encephalomyelitis, which is a test procedure emulating multiple sclerosis, an autoimmune disease. The procedures used and the results obtained are briefly described below.

The PHA-stimulated PMBC proliferation standard pharmaceutical test procedure was used to evaluate the immunosuppressive activity of the combination of the rapamycin and $1,25(OH)_2D_3$ combination. Briefly, human PBMC were isolated from freshly collected heparinized venous blood of healthy volunteers by Ficoll-Hypaque density gradient centrifugation. Cells were resuspended at a density of 10^6/ml in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and mycostatin (100 U/ml), and were cultured in 5% $C0_2$ / 95% air at 37•C with phytohaemagglutinin (PHA, 0.1 µg/ml), $1,25(OH)_2D_3$ (10^{-9} to 3.125×10^{-6} M), rapamycin (0.32 to 1000 ng/ml, i.e. 3.501×10^{-10} to 1.094×10^{-6} M), excipient control or combinations of serial dilutions of $1,25(OH)_2D_3$ and rapamycin. Flat bottomed multiwell plates (NUNC, Denmark) were used, containing 0.2 ml of final cell suspension in each well. Rapamycin and $1,25(OH)_2D_3$ were always added to PBMC cultures 20 to 30 min before PHA. Each individual concentration or combination, as well as the control were tested in quadruplicate. After 48 hours of incubation, each well was pulsed with 0.5 pCi of [^{3}H]-thymidine. The plates were reincubated for another 18 hours, and then harvested using a micro cell harvester. Radioactivity was measured with a β-scintillation counter, and the percent proliferation was calculated.

The results obtained in this standard pharmacological test procedure are provided in the table below, which shows the dosages necessary to produce specified percent inhibition of PHA-stimulated PMBC proliferation.

PERCENT INHIBITION OF PMBC PROLIFERATION

% Inhibition	Monotherapy Rapamycin (ng/ml)	Monotherapy $1,25(OH)_2D_3$ (M)	Combination Therapy Rapamycin (ng/ml)	Combination Therapy $1,25(OH)_2D_3$ (M)
20	2.2	4.7×10^{-9}	0.0034	1.1×10^{-11}
40	49	1.9×10^{-6}	0.27	8.5×10^{-10}
50	180	2.2×10^{-5}	1.7	5.2×10^{-9}
60	640	2.6×10^{-3}	10.1	3.2×10^{-8}
80	1440	0.1	810.9	2.5×10^{-6}

These results show that rapamycin, $1,25(OH)_2D_3$, and the combination of rapamycin and $1,25(OH)_2D_3$ all inhibited PHA-stimulated PMBC proliferation. Rapamycin alone had an IC_{50} of 180 ng/ml, $1,25(OH)_2D_3$ alone had an IC_{50} of

2.2×10^{-5} M, whereas an IC_{50} was achieved for the combination of rapamycin and $1,25(OH)_2D_3$ at a dose of 1.7 ng/ml rapamycin and 5.2×10^{-9} M $1,25(OH)_2D_3$. The combination gave 50% suppression at doses more than 100 and 4000 fold lower than for rapamycin and $1,25(OH)_2D_3$ monotherapy, respectively. These results show that the combination of rapamycin and $1,25(OH)_2D_3$ acted synergistically as an immunosuppressive agent.

The experimental allergic encephalomyelitis (EAE) standard pharmacological test procedure was used to further evaluate the immunosuppressive activity of the combination of rapamycin and $1,25(OH)_2D_3$. This test procedure also evaluated the combination's activity against autoimmune disorders. Briefly, mouse spinal cord, isolated from NMRI mice, was lyophilized and stored as powder at +4°C. The mice were injected in their hind foot pads with 50 µl of an emulsion consisting in an equal volume mixture of mouse spinal cord in PBS (55 mg/ml) and mycobacterium tuberculosis strain H37-Ra in complete Freund's adjuvant (CFA, 4 mg/ml). The induction day was considered day 0. On days 0 and +2, pertussis toxin, 200 ng in a volume of 50 µl PBS, was given intravenously on the induction day and repeated two days later.

Control mice (group CTR = 42 mice) were induced and injected daily with 100 µl of the rapamycin vehicle (0.2% carboxymethylcellulose in phosphate buffered saline), and every two days with the $1,25(OH)_2D_3$ vehicle (100 µl of peanut oil). The next three groups received only $1,25(OH)_2D_3$ at 1 1µg/kg (group D1 =21 mice), 2 1µg/kg (group D2 = 23 mice), and 5µg/kg (group D5 = 20 mice), on alternate days, starting with day -3 (three days before induction). The rapamycin alone treated groups were injected either daily, with 0.1 mg/kg (group RP0.1 = 25 mice), 0.3 mg/kg (group RP0.3 = 36 mice), 0.6 mglkg (group RP0.6 = 32 mice) and 1 mg/kg (group RP1 = 28 mice), or every two days, with 0.6 mg/kg (group RP0.6' = 35 mice). The last two groups were treated with combinations between $1,25(OH)_2D_3$ at 2 pg/kg every 2 days and the same total dose of rapamycin, but given either in daily administrations of 0.3 mg/kg (group MIX-1, 25 mice), or 0.6 mg/kg given every two days (group MIX-2, 23 mice). The administration of rapamycin in the last group was alternated with that of $1,25(OH)_2D_3$, starting with rapamycin on day -3. Three to seven urine samples were collected from each mouse which measured urinary calcium and collagen cross-links

content (deoxypyridinoline, DPD). All mice were sacrificed on day 20, or earlier if severely paralyzed. Blood was collected by heart puncture, under ether anesthesia. Serum was stored at -20°C, for further measurement of calcium and osteocalcin. Duodenum, 1 cm below the pylorus, was isolated, rinsed and stored at -20°C, until assayed for calbindin D-9K content. Brain and spinal cord were removed and fixed in Bouin solution for histology.

Mice were assessed for clinical signs of paralysis, starting on day 9. EAE signs were scored daily, on a scale from 0 to 4. Scores were evaluated blindly, by two independent observers. To be considered positive for clinical EAE, a mouse must have been scored 0.5 or higher for 2 consecutive days. For the mice found dead after previously presenting signs of disease, or for moribunds sacrificed before day 20, a score of 4 was considered from the day of their death until the end of the follow up. For comparing the disease evolution of different groups, the disease free survivorship, representing the number of disease free mice of one group on the day of sacrifice, and the following parameters of disease severity were used: the cumulative score, defined as the sum of the daily mean scores of one group between days 11 and 20 of the follow up; the integrated disease score, calculated by making the mean of all individual daily scores of the last 10 days for one group, and the mean maximal disease score, which is the mean of the maximal disease scores reached by all the mice of one group.

The results of this standard pharmacological test procedure showed that treatment with both $1,25(OH)_2D_3$ and rapamycin alone significantly decreased disease severity ($p < 0.0001$ by ANOVA test). All but one of the control mice showed signs of paralysis. Disease onset was observed between 9 and 15 days post induction (mean 12.1 ± 0.3 days). The disease generally became more severe, until reaching a peak score in 1 to 4 days, then the mice either died or slowly recovered. Similar evaluations were noticed in the groups treated with the lowest doses of $1,25(OH)_2D_3$ and RAP (groups D1 and RPO.1). Higher drug doses manifested more substantial protective effects. In the group treated with $1,25(OH)_2D_3$ at 2 μg/kg every 2 days, 8 of 23 mice were disease free at the end of the follow up (30 %, group D2), whereas 5 μg/kg every 2 days prevented the appearance of disease in 65 % of the treated mice (13 out of 25 mice, group D5) and postponed the disease onset in the mice who did develop the disease ($p < 0.01$). Rapamycin at 1 mg/kg/day (group RP1) conferred near to total protection (only 2 out of 28 animals developed mild disease), whereas lower doses were only partially protective. For instance, rapamycin at 0.3 mg/kg/day prevented disease evolution in 45 % of the animals (group RP0.3), while the same drug quantity

given every two days (0.6 mg/kg every 2 days, group RP0.06') was somehow less protective (31 % disease free animals at the end of the observation period).

The combination of subtherapeutic doses of $1,25(OH)_2D_3$ and rapamycin (groups MIX-1 and MIX-2) provided near total protection, comparable to that obtained in the group RP1. The protection achieved with the combination treatments was by far more efficient than that obtained with each drug given separately.

These results are summarized in the table below.

CLINICAL EVALUATION OF EAE

Group	Percent Disease Free	Mean Day of Onset	Mean Maximal Score
CTR - placebo	2.4	12.1 ± 0.3	3.2 ± 0.2
D1 - $1,25(OH)_2D_3$ 1µg/kg /2 days	19	13 ± 0.5	2.7 ± 0.4
D2 - $1,25(OH)_2D_3$ 2µg/kg /2 days	30	13 ± 0.4	2.1 ± 0.3
D5 - $1,25(OH)_2D_3$ 5µg/kg /2 days	65	15.3 ± 0.7	1.1 ± 0.4
RP0.1 - RAP 0.1 mg/kg /day	24	12.1 ± 0.5	2.5 ± 0.3
RP0.3 - RAP 0.3 mg/kg /day	45	12.5 ± 0.5	1.6 ± 0.3
RP0.6 - RAP 0.6 mg/kg /day	75	11.1 ± 0.5	0.8 ± 0.2
RP1 - RAP 1 mg/kg /day	94	14	0.2 ± 0.1
RP0.6' - RAP 0.6 mg/kg /2 days	31	13.9 ± 0.3	2.2 ± 0.3
MIX1 - $1,25(OH)_2D_3$ 2µg/kg + RAP 0.3 mg/kg	92	16.5 ± 0.5	0.28 ± 0.2
MIX2 - $1,25(OH)_2D_3$ 2µg/kg + RAP 0.6 mg/kg, alt	87	15.7 ± 0.9	0.28 ± 0.2

The severity of brain and spinal cord inflammation corresponded in general to the clinical score at the sacrifice day for each mouse. Moderate to severe mononuclear infiltration was observed in seven out of the eight control mice, with mild inflammation still present in the eighth. The number of mice with mild or no central nervous system inflammation were four, five and three out of eight for groups D2, RP0.3 and RP0.6' respectively. In both combination treated groups, as well as in group RP1, seven out of eight mice were inflammation - free, only one showing mild mononuclear infiltration. Mean histological score was significantly lower than that of group D2 in both combination groups. The score of group MIX-2 was also significantly lower than that of group RP0.6', whereas the difference between the scores of groups MIX- 1 and RP0.3 reached the significance limit ($p = 0.06$).

These results are summarized in the table below.

MEAN HISTOLOGICAL SCORE AND INFLAMMATION

Group	Mean Histological Score	Inflammation*
CTR - placebo	1.75 ± 0.18	8 / 8
D1 - 1,25$(OH)_2D_3$ 1μg/kg /2 days		
D2 - 1,25$(OH)_2D_3$ 2μg/kg /2 days	0.88 ± 0.23	8 / 8
D5 - 1,25$(OH)_2D_3$ 5μg/kg /2 days		
RP0.1 - RAP 0.1 mg/kg /day	0.97 ± 0.34	7 / 8
RP0.3 - RAP 0.3 mg/kg /day	0.88 ± 0.35	6 / 8
RP0.6 - RAP 0.6 mg/kg /day	0.22 ± 0.12	3 / 8
RP1 - RAP 1 mg/kg /day	0.03 ± 0.03	1/8
RP0.6' - RAP 0.6 mg/kg /2 days	1.19 ± 0.3	7 / 8
MIX1 - 1,25$(OH)_2D_3$ 2μg/kg + RAP 0.3 mg/kg	0.13 ± 0.13	1 / 8
MIX2 - 1,25$(OH)_2D_3$ 2μg/kg + RAP 0.6 mg/kg, alt	0.09 ± 0.09	1 / 8

* Number of animals with CNS inflammation.

The results obtained in this standard pharmacological test procedure showed that the combination of rapamycin and 1,25$(OH)_2D_3$ acted synergistically as an immunosuppressive agent and in inhibiting the formation of experimental allergic encephalomyelitis, a standard model of autoimmune disorders.

Based on the results obtained in the standard pharmacological test procedures the combination of rapamycin and 1,25$(OH)_2D_3$ is useful in treating or preventing diseases or disorders involving the immune system. These include, but are not limited to, in the treatment or inhibition of transplantation rejection such as kidney, heart, liver, lung, bone marrow, pancreas (islet cells), cornea, small bowel, and skin allografts, and heart valve xenografts; treatment or inhibition of autoimmune diseases and diseases of inflammation such as systemic lupus erythematosis, rheumatoid arthritis, diabetes mellitus, myasthenia gravis, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, scleroderma, Wegener's granulomatosis, chronic active hepatitis, biliary cirrhosis, sarcioidosis, nephrotic syndrome, multiple sclerosis, Steven-Johnston syndrome, psoriasis, dermatitis, eczema, seborrhea, idiopathic sprue, Crohn's disease, inflammatory bowel disease, Graves ophthalmopathy, and interstitial

lung fibrosis; ocular inflammation, such as eye uveitis; pulmonary inflammation such as asthma, chronic obstructive pulmonary disease, emphysema, acute respiratory distress syndrome, and bronchitis; and hyperproliferative vascular disorders such as restenosis vascular occlusion, particularly following either biologically or mechanically mediated vascular injury leading to intimal smooth muscle cell proliferation. The combination of rapamycin and $1,25(OH)_2D_3$ is also useful in treating fungal infections and adult T-cell leukemia/lymphoma, and as an antineoplastic agent.

It is anticipated that the combination of rapamycin and $1,25(OH)_2D_3$ may be used in combination with other agents useful in treating or preventing diseases or disorders involving the immune system, such as azathioprine, corticosteroids, such as prednisone and methylprednisolone, cyclophosphamide, cyclosporin A, FK-506, OKT-3, and ATG to further reduce dosage requirements needed to achieve the desired effect.

When the combination of rapamycin and $1,25(OH)_2D_3$ is employed to induce immunosuppression, it can be formulated neat or with a pharmaceutical carrier to a mammal in need thereof. The pharmaceutical carrier may be solid or liquid.

A solid carrier can include one or more substances which may also act as flavoring agents, lubricants, solubilizers, suspending agents, fillers, glidants, compression aids, binders or tablet-disintegrating agents; it can also be an encapsulating material. In powders, the carrier is a finely divided solid which is in admixture with the finely divided active ingredient. In tablets, the active ingredient is mixed with a carrier having the necessary compression properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain up to 99% of the active ingredient. Suitable solid carriers include, for example, calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, methyl cellulose, sodium carboxymethyl cellulose, polyvinylpyrrolidine, low melting waxes and ion exchange resins.

Liquid carriers are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The active ingredient can be dissolved or suspended in a pharmaceutically acceptable liquid carrier such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid carrier can contain other suitable pharmaceutical additives such as solubilizers, emulsifiers, buffers, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, colors, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid carriers for oral and parenteral administration include water (partially

containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the carrier can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid carriers are useful in sterile liquid form compositions for parenteral administration. The liquid carrier for pressurized compositions can be halogenated hydrocarbon or other pharmaceutically acceptable propellant.

Liquid pharmaceutical compositions which are sterile solutions or suspensions can be utilized by, for example, intramuscular, intraperitoneal or subcutaneous injection. Sterile solutions can also be administered intravenously. The compound can also be administered orally either in liquid or solid composition form.

The combination of rapamycin and $1,25(OH)_2D_3$ may be administered rectally in the form of a conventional suppository. For administration by intranasal or intrabronchial inhalation or insufflation, the compounds of this invention may be formulated into an aqueous or partially aqueous solution, which can then be utilized in the form of an aerosol. The combination of rapamycin and $1,25(OH)_2D_3$ may also be administered transdermally through the use of a transdermal patch containing the active compound and a carrier that is inert to the active compound, is non toxic to the skin, and allows delivery of the agent for systemic absorption into the blood stream via the skin. The carrier may take any number of forms such as creams and ointments, pastes, gels, and occlusive devices. The creams and ointments may be viscous liquid or semisolid emulsions of either the oil-in-water or water-in-oil type. Pastes comprised of absorptive powders dispersed in petroleum or hydrophilic petroleum containing the active ingredient may also be suitable. A variety of occlusive devices may be used to release the active ingredient into the blood stream such as a semipermiable membrane covering a reservoir containing the active ingredient with or without a carrier, or a matrix containing the active ingredient. Other occlusive devices are known in the literature.

The combination of rapamycin and $1,25(OH)_2D_3$ may be administered topically as a solution, cream, or lotion by formulation with pharmaceutically acceptable vehicles containing 0.1 - 5 percent, preferably 2%, of active compound.

The dosage requirements vary with the particular compositions employed, the route of administration, the severity of the symptoms presented and the particular subject being treated. Based on the results obtained in the standard pharmacological test procedures, projected daily intravenous dosages of the combination of rapamycin and $1,25(OH)_2D_3$ would be 0.001 - 25 mgkg rapamycin and 0.001 - 50 μg $1,25(OH)_2D_3$.

Projected daily oral dosages of the combination of rapamycin and $1,25(OH)_2D_3$ would be 0.01 - 50 mg/kg rapamycin and 0.01 - 100 μg $1,25(OH)_2D_3$.

Treatment will generally be initiated with small dosages less than the optimum dose of the compound. Thereafter the dosage is increased until the optimum effect under the circumstances is reached; precise dosages for oral, parenteral, intranasal, intrabronchial, transdermal, or rectal administration will be determined by the administering physician based on experience with the individual subject treated. In general, the combination of rapamycin and $1,25(OH)_2D_3$ is most desirably administered at a concentration that will generally afford effective results without causing any harmful or deleterious side effects.

WHAT IS CLAIMED IS:

1. A method of inducing immunosuppression in a mammal in need thereof which comprises, administering a combination of rapamycin and 1,25-dihydroxy-cholecalciferol to said mammal orally, parenterally, intranasally, intrabronchially, transdermally, or rectally.

2. A method of inhibiting or treating organ or tissue transplantation rejection in a mammal in need thereof which comprises, administering a combination of rapamycin and 1,25-dihydroxycholecalciferol to said mammal orally, parenterally, intranasally, intrabronchially, transdermally, or rectally.

3. The method according to claim 2 wherein the transplanted organ or tissue is selected from the group consisting of kidney, heart, liver, lung, bone marrow, pancreas (islet cells), cornea, small bowel, skin, and heart valve.

4. A method of treating or inhibiting autoimmune disorders in a mammal in need thereof which comprises administering a combination of rapamycin and 1,25-dihydroxycholecalciferol to said mammal orally, parenterally, intranasally, intrabronchially, transdermally, or rectally.

5. A method of treating or inhibiting inflammatory diseases or disorders which comprise administering a combination of rapamycin and 1,25-dihydroxycholecalciferol to said mammal orally, parenterally, intranasally, intrabronchially, transdermally, or rectally.

6. A method of treating or inhibiting hyperproliferative vascular disorders, which comprises administering a combination of rapamycin and 1,25-dihydroxycholecalciferol to said mammal orally, parenterally, intranasally, intrabronchially, transdermally, or rectally.

7. A method of treating or inhibiting neoplasms which comprises administering a combination of rapamycin and 1,25-dihydroxycholecalciferol to said mammal orally, parenterally, intranasally, intrabronchially, transdermally, or rectally.

8. A pharmaceutical composition which comprises rapamycin, 1,25-dihydroxycholecalciferol and a pharmaceutical carrier.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/19378

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/435 A61K31/045 //(A61K31/435,31:045)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BRANISTEANU, DUMITRU D. ET AL: "Synergism between sirolimus and 1,25-dihydroxyvitamin D3 in vitro and in vivo" J. NEUROIMMUNOL. (1997), 79(2), 138-147 CODEN: JNRIDW;ISSN: 0165-5728, XP002053686 see abstract	1-8
Y	---	1
X	SHANE ET AL: "IMMUNOSUPPRESIVE THERAPY AND THE SKELETON" TRENDS ENDOCRINOL. METAB., vol. 5, no. 4, 1994, USA, pages 169-175, XP002053687 see page 169, left-hand column, line 1 - middle column, line 10 see page 172, middle column, line 13-15	1-8

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
28 January 1998	06.02.98
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	**Authorized officer** Herrera, S

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/19378

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOFFE, IAN ET AL: "Lack of change of cancellous bone volume with short-term use of the new immunosuppressant rapamycin in rats" CALCIF. TISSUE INT. (1993), 53(1), 45-52 CODEN: CTINDZ;ISSN: 0171-967X, XP002053688 see abstract	1-8
Y		1

Y	US 5 286 730 A (CAUFIELD CRAIG E ET AL) 15 February 1994 cited in the application see abstract	4-6

Y	EP 0 525 960 A (AMERICAN HOME PROD) 3 February 1993 cited in the application see abstract	6,7

Y	GARDNER C.R.: "THE PHARMACOLOGY OF IMMUNOSUPPRESSANT DRUGS IN SKIN TRANSPLANT REJECTION IN MICE AND OTHER RODENTS" GENERAL PHARMACOLOGY, vol. 26, no. 2, 1995, UK, pages 245-271, XP002053689 see page 257, right-hand column, line 10-51 see page 260, right-hand column, line 34-38	1-3

Y	RIGBY W.F.C.: "THE IMMUNOBIOLOGY OF VITAMIN D" IMMUNOL. TODAY, vol. 9, no. 54, 1988, HOLLAND, pages 54-58, XP002053690 cited in the application see page 57, left-hand column, line 45-47 see page 57, right-hand column, line 8-20	1,4,5

Y	LEMIRE J M: "IMMUNOMODULATORY ROLE OF 1,25-DIHYDROXYVITAMIN D3" JOURNAL OF CELLULAR BIOCHEMISTRY, vol. 49, no. 1, May 1992, pages 26-31, XP000653445 cited in the application see abstract	1-4,6,7

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INTERNATIONAL SEARCH REPORT

...rnational application No.
PCT/US 97/ 19378

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-7
because they relate to subject matter not required to be searched by this Authority, namely:
SEE FURTHER INFORMATION SHEET PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 97/19378

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

Claims Nos.: 1-7

because they relate to subject matter not required to be searched by this Authority, namely:

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

Remark : Although claims 1-7
are directed to a method of treatment of
the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/19378

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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